Expression of Acetyl Coa Carboxylase Gene from *Cleome Viscosa* L. and Its Seed Oil

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Abstracts: Asian spider flower (*Cleome viscosa* L.) is annual herbaceous weed plant. Its seed oil has similar properties to the seed oil from *Jatropha curcas* L. which is the biodiesel plant. This research focused on the expression of acetyl CoA carboxylase gene in 4 stages of seeds (1-4 weeks after fruit set (WAF)) by Reverse transcriptase Polymerase Chain Reaction (RT-PCR). Acetyl CoA carboxylase (ACCase) is the essential enzyme for the first step in the biosynthesis of long-chain fatty and glycerol. The heteromeric ACCase consists of 4 subunits. *acc*A, *acc*B and *acc*C gene were located in nuclear, while the *acc*D gene was in chloroplast. The result showed that the expression of *acc*A gene showed the most highly expressed in 3 WAF, while the expression of *acc*C and *acc*D showed the most highly expression in 2-3 WAF. These results indicated that co-expression of *acc*A, *acc*C and *acc*D were highest in 3 WAF and down regulated in 4 WAF. In contrast, *acc*B gene showed the high expression level in 4 weeks after fruit set. The total oil was obtained by petroleum ether soxhlet extraction. The high oil content was 3-4 WAF. These findings suggested that comparatively high expression of the Accase gene related to the persistence of oil accumulation during the late stage of seed oil formation for *C. viscosa* L.

Keywords: *Cleome Viscosa* L., Acetyl CoA Carboxylase Gene, Expression of Gene, Oil Content.

1. INTRODUCTION

The biofuel and biodiesel are the renewable fuel produced from plants or plant-derived materials. Reducing CO2 emissions through the use of biofuels and biodiesel is an efficient strategy to fight global climate change. Plant species which were used in biodiesel production in Thailand were oil palm (*Elaeis guineensis* Jacq), physic nut (*Jatropha curcas* L.), soybean (*Glycine max* (L.) Merr.), rice (*Oryza sativa* L.), coconut (*Cocos nucifera* L.). However, biodiesel production in Thailand was still insufficient to meet the demand because of the expanding of economic growth. The Ministry of Energy of Thailand has stipulated that biodiesel blends are 7%, and palm oil were used as the main raw material. However, most of the raw materials for biodiesel production have still imported from abroad. This made the government and industry sectors turn to promote more plantations of oil palm and black soap in Thailand. However, palm oil and physic nut were still experiencing problems in terms of being a perennial plant. This requires quite a lot of space for planting to produce high yields. Seed oil of Asian spider flower (*Cleome viscosa* L.) has similar property to physic nut [1]. It has used less space for planting and relatively low-cost production. The oil content per weight of this plant was quite high and there was no competition for food production. Major *fatty acids* were identified as linoleic acid, *palmitic* acid, *stearic* acid, *oleic* acid, and linolenic [2 and 3]. Therefore, its seed has a potential for biodiesel production.

De novo fatty acid synthesis (FAS) pathway in plants occurs in the plastids. Acetyl-CoA carboxylase (ACCase) is the enzyme that catalyzes the first step in fatty acid biosynthesis formation of malonyl-CoA from acetyl-CoA. There are two structurally distinct forms, Accase homomeric composed of single subunit in plant, yeast and animals [4]. Heteromeric or multisubunit ACCase is found in plastids of most plants and prokaryote. The heteromeric ACCase of higher plants compose of four subunits; the α-subunit of carboxyltransferase (α-CT, encoded by accA gene), a biotin carboxyl carrier protein (BCCP, encoded by accB gene), a biotin carboxylase (BC, encoded by accC gene) and a β-subunit of carboxyltransferase (β-CT, encoded by accD gene). The accD gene is located in chloroplasts, while the accA, accB and accC genes are the nucleus [5, 6 and 7]. The expression of ACCase genes in Jatropha was correlated with the free fatty acid content obtained by GC (Gas Chromatography) analysis [8] (Booranasrisak et al. 2013). Studies on the structure and cloning of ACCase gene in several plants such as Arabidopsis [9], *Brassica napus* [10] and oil palm [11] found that this gene was expressed in cells or tissues accumulation lipid. In addition,

Turnham and Northcote (1983) [12] studied the relationship between ACCase activity and lipid accumulation during seed development of rapeseed (*Brassica napus*) after 5-34 days of pollination. Activity of the ACCase enzyme was not correlated with lipid synthesis during embryo development. However, over-expression of the ACCase from Arabidopsis in transgenic potato tubers led to an increase in fatty acid synthesis [13]. This study is focusing on identification of differentially expressed genes and assessment of the gene expression patterns in developing seeds. This will help identify which candidate genes have function in oil accumulation.

2. MATERIEL AND METHODS

2.1 Plant Samples

Asian spider flower (*C. viscosa* L.) seeds were collected from various locations around the Naresuan University and grown in pots located in plant nursery at the Department of Biology, Faculty of Science, Naresuan University. Four different stages of fruit were 1-4 weeks after fruiting (WAF) and young leaves at shoot tips.

2.2 RNA Extraction

Young leaves were used for total RNA extraction by LiCl methods [14] and NucleoSpin® Plant and Fungi kit was used for total RNA extraction from 4 different stages of seeds. The quality of the total RNA was verified by electrophoresis with 1.2% agarose gel and total RNA quantification were verified by ultraviolet absorbance measurements at 260 and 280 nm wavelengths using a fine-grained genome spectroscopy (Nanadrop).

2.3 First Strand cDNA Synthesis

Total RNA was extracted from young leaves and seeds of Asian spider flower were used for first strand cDNA synthesis with Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). To digest DNA, RNA template, 10X Reaction Buffer with $MgCl_2$, DNaseI, Deionized water were mixed and incubated at 37 °C for 60 minutes. Then adding 1 µl of 50 mM EDTA and 1 µl of Oligo $(dT)_{18}$ before spined down the solution and incubated at 65 °C for 5 minutes. Then adding 5X Reaction Buffer, RiboLock RNase Inhibitor, 10 mM dNTP Mix, RevertAid M-MuLV RT and incubated at 42 °C for 60 minutes. Stop the reaction at 70 °C for 5 minutes.

2.4 Cloning and Sequencing

Primers were designed in conserved gene regions based on the homology of *ACCase* sequences in the National Center for Biotechnology Information (NCBI) GenBank database and aligned with the Clustal X software (v.2.0). The PCR amplification was performed using Phusion™ High-Fidelity DNA polymerase (Thermo Scientific, USA). The purified PCR products were cloned into the pJET1.2/blunt cloning vector using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific, USA) and sequenced. Next, cDNA sequences were analyzed using the Gene studio software and compared to their corresponding sequences using the NCBI BLAST tool. Partial sequences had been deposited in the NCBI GenBank database and used these data for designed the real time PCR primers.

2.5 Amplification of ACCase Genes

ACCase genes were *accA*, *acc*B, *acc*C and *acc*D which amplified from cDNA. PCR was conducted using the following program: 94 °C for 5 min followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min. Products of PCR were separated on 1.0% agarose gel and the DNA was stained with ethidium bromide.

2.6 Expression of ACCase Genes

Expression levels of the 4 genes of ACCase genes were verified in young leaves and 4 different stages of seeds of Asian spider flower by real-time quantitative PCR (qPCR). The qPCR was performed using SensiFASTTM SYBR

No-ROX Kit (Bioline, UK). Selected fluorescence labeling primers of *acc*A, *acc*B, *acc*C, and *acc*D genes were used for amplified ACCase genes by LightCycler ® 480 Instrument II (Roche, Switzerland). To check reproducibility, each assay was performed with technical triplicates for each of the three biological samples. The PCR expression pattern was analyzed from the exponential phase curve data and the specificity of amplification PCR associated with gene expression were examined by using the delta-delta-Ct ($2^{-\Delta\Delta ct}$) method [10] which used *actin* as reference gene.

2.7 Statistical Analysis

All genes expression of each treatment were analyzed by the Duncan One Way ANOVA test using SPSS program. To compare the differences between treatments where all data is normalized and a significant difference at $P \leq 0.05$.

2.8 The Crude Oil Yield

The seed oil content was analyzed by the Soxhlet extraction method. Seeds from four different stages of *C. viscosa* L. (1-4 WAF) dried at room temperature for 7 days. 4 g of seeds were grinded into fine powder and transferred into a Soxhlet apparatus and extracted with petroleum ether (boiling point under 60-80°C) for 8 h. After extraction, the solvent was removed using rotary evaporator for 30 min and dried by hot air-oven at 103°C for 15 min and then weighed. The crude oil percentages were calculated by below formula:

(total oil weight/ 4 g of seeds) X 100.

3. RESULTS

3.1 RNA Extraction

Young leaves were used for total RNA extraction by LiCl methods [14] and NucleoSpin® Plant and Fungi kit was used for total RNA extraction from 4 different stages of seeds. These 2 methods can extract total RNA from *C. viscosa* samples. However, total RNA in lanes 3.1, 3.2 and 4.1, 4.2 were lesser amount when compared to other samples (Figure 1). This might cause by the seed maturation. The mature seeds are not only harder seedcoat than younger seed but also have high phenolic compound contents which might interfere the total RNA extraction.

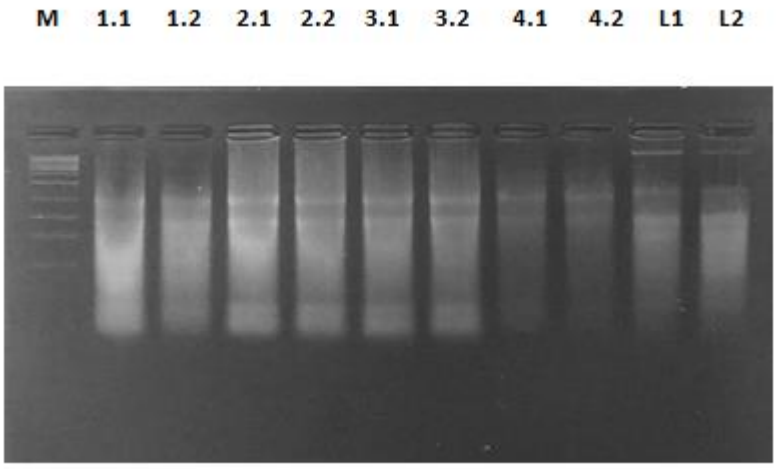

Figure 1. Total RNA extraction of *C. viscosa* samples M=DNA Marker hyper ladder 1 kp, 1.1, 1.2 = 1 WAF, 2.1, 2.2 = 2 WAF, 3.1, 3.2 = 3 WAF, 4.1, 4.2 = 4 WAF and L1, L2 = young leaves at shoot tips.

3.2 Cloning and Sequencing

The partial DNA sequences from each colony were edited and aligned using GeneStudio™ software (GeneStudio, Inc, USA) and the ClustalW (EMBL-EBI, UK) respectively. The partial sequences of *C. viscosa* Accase genes (*acc*A, *acc*B, *acc*C and *acc*D) were deposited in the DDBJ/EMBL/GenBank DNA database and the accession numbers were obtained as *acc*A (OM459998), *acc*B (OM459999), *acc*C (OM460000) and *acc*D (OM460001). The length of *acc*A, *acc*B, *acc*C and *acc*D were 340, 473, 276 and 422, respectively. These genes show > 75% highly similarity with *Tarenaya hassleriana*, *Camelina sativa*, *Brassica oleracea* and *Capsella rubella*.

3.3 Expression of ACCase genes

The *acc*A, *acc*B, *acc*C and *acc*D gene expression were examined three times. Each time, a sample of *C. viscosa* L. from different locations was used for examination of the gene expression and used these data for statistical analysis. The *acc*A expression in seeds was highest at 3 weeks after fruiting (WAF) and was 177.47 times. Specifically, accA expression in seeds from 3 WAF was significantly higher than that of seeds from 1 WAF and 4 WAF. However, there was no difference between stages 3 and 2 WAF (Fig. 2 A). The expression of the *acc*B gene of seed from 4 WAF was the highest gene expression at 23.53 times but there was no significant difference among seed stages (Fig. 2 B). The highest *acc*C gene expression was found in the post-fruiting period, between 2-3 weeks and were 6.11 and 6.95 times, respectively. There was also no significant difference among seed stages (Fig. 2 C). The highest *acc*D gene expression was found in the post-fruiting period between 2-3 weeks and were 1.95 and 1.96 times, respectively, with no significant differences among seed stages (Fig. 2 D). The *acc*A, *acc*C, and *acc*D genes were highly expressed in seeds 2-3 WAF. On the other hand, the *acc*B gene was differing from the *acc*A, *acc*C and *acc*D genes which was highly expressed at 4WAF, and there was also a difference in each stage. The total oil in the four seed developmental stages (1-4 WAF) was determined. The seed oil yield in 1-4 WAF were 2.87±0.93, 12.98±1.18%, 21.07±0.77% and 23.95±1.81%, respectively. However, there was no significant difference between seed from 3 and 4 WAF stages (Fig. 3 and Table 1). The oil yield increased almost linearly during the seed developmental period and reached the maximal level in the last stage (4 WAF).

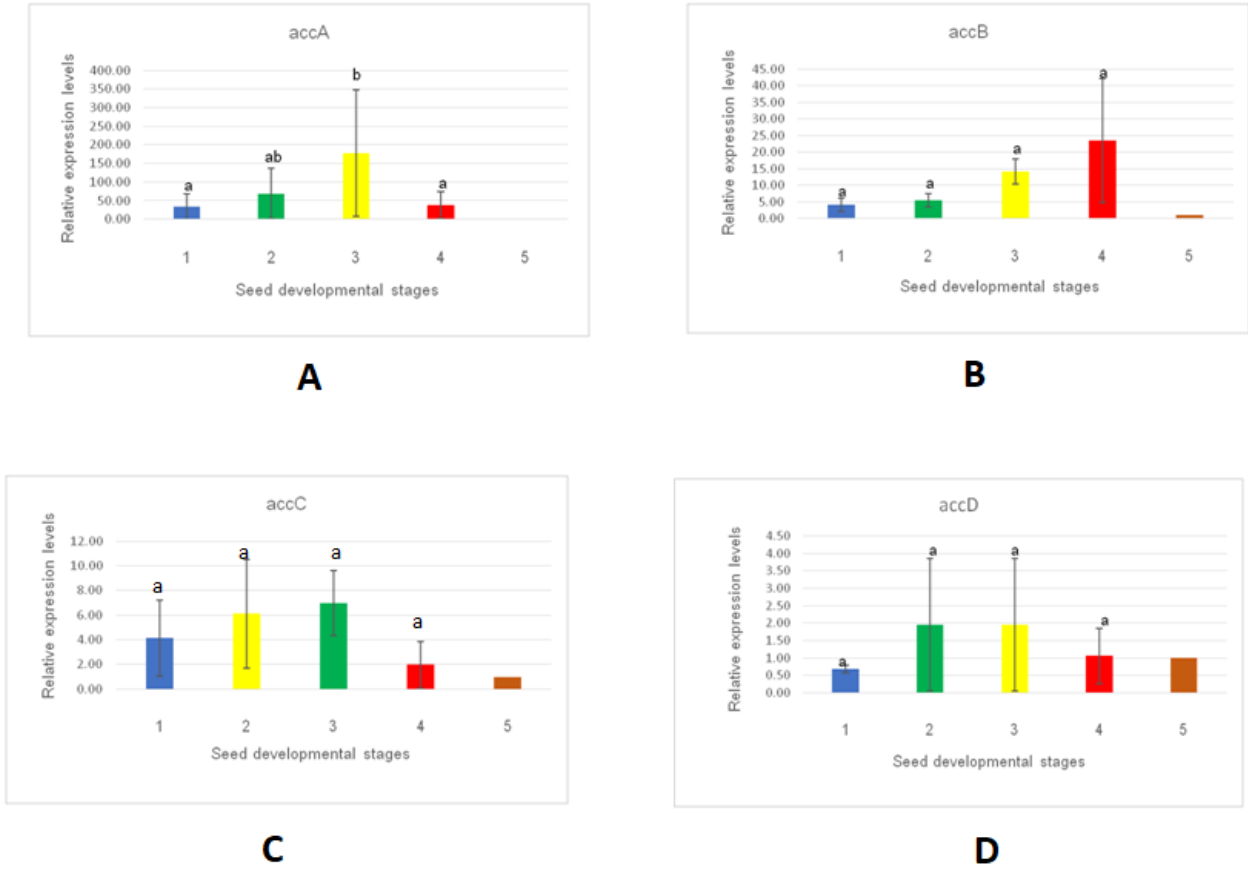

Figure 2. Expression of acetyl CoA carboxylase genes in different stages of seed and young leaves. Acetyl CoA carboxylase gene expression of real-time PCR analysis (A-D), (A) *acc*A, (B) *acc*B, (C) *acc*C and (D) *acc*D, 1 = 1 week after fruiting, 2 = 2 weeks after fruiting, 3 = 3 weeks after fruiting, 4 = 4 weeks after fruiting and 5= young leaves.

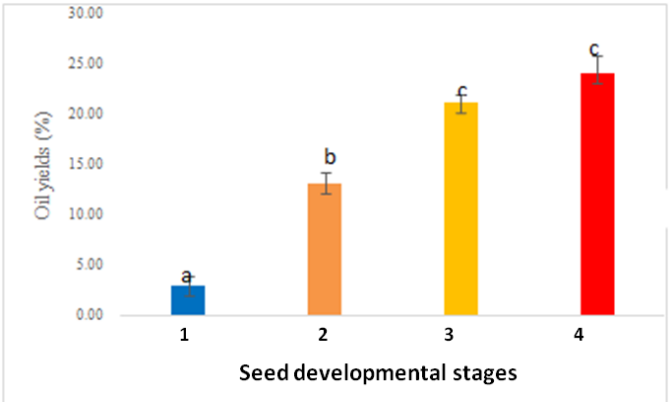

Figure 3 Crude oil yield in different stages of seed. 1 = 1 week after fruiting, 2 = 2 weeks after fruiting, 3 = 3 weeks after fruiting, 4 = 4 weeks after fruiting and 5= young leaves

Table 1. Oil yield of *C. viscosa* L. seed in different stages.

Seed stages (weeks after fruiting)	Oil yield (%)
1	2.87±0.93a
2	12.98±1.18b
3	21.07±0.77c
4	23.95±1.81c

4. DISCUSSION

The expression of *acc*A, *acc*C, and *acc*D genes were high in seed stages 2-3 WAF of C. *viscosa* L. which are in middle period of fruit development. This corresponds to the gene study in *Jatropha* [15] which were highly expressed in the middle period of the fruit development. This phase was the fatty acid and glycerol synthesis reactions which had occurred before entering to the oil deposition state. However, the *acc*B gene was highly expressed at 4 WAF which was differing from the *acc*A, *acc*C and *acc*D genes. This was consistent with the study of the structure of the heteromeric ACCase genes, that can be found in prokaryotes and the plastids of most plants. The *acc*A, *acc*C and *acc*D genes were catalytic domains, while the *acc*B gene was structural domains, which are structurally different from the three genes [16]. According to the genome information, the *Arabidopsis* genome contains 1-2 genes that encode the heteromeric ACCase, two genes for *acc*B and one gene for each *acc*A, *acc*C and *acc*D [17]. Microarray analyses of gene expression in Arabidopsis seeds, the ACCase subunit was shown to be expressed in Arabidopsis seeds between days 8 - 11, which is the intermediate stage of seed maturity [18]. In the middle of the seed of *C. viscosa*, the *acc*A, *acc*C, and *acc*D genes were also expressed. Additionally, it was demonstrated that the heteromeric ACCase component ß-CT (*acc*D gene) in oil palm and rapeseed (*Brassica napus*) increased the rate of ACCase catalysis concurrently with seed maturity from day 18 - 22, the amount of fat increases rapidly before dropping out at a constant level. However, the activity of ACCase enzyme was lowest at day 34 during embryogenesis in tissue culture of oil palm [12]. This was in accordance with the effect of gene expression on *acc*D observed in this study. In the middle stage of seed development, gene expression was high similar to rapeseed. Moreover, the overexpression of *acc*D gene had increased the tobacco seed yields and improved seed oil contents [6].

C. viscosa L. from different areas in India which are Delhi, Faridabad, Surajkund, Jaipur, and Hyderabad have oil yields varied between 21-26%. This is in the same range of oil percentage from our study. The seed oil of *C. viscosa* L. has a similar fatty acid composition to edible oil plants such as soybean, sunflower, safflower, linseed, and rapeseed as well as non-edible oils from rubber, Jatropha, and Pongamia [19] . According to the study by Bhardwaj and Hamama (2003) [20] in rapeseed, from the early stages of seed growth through the late stages, the lipid content gradually increased. On the other hand, the accumulation of crude oil in the growing sunflower seeds started at the beginning of seed development, increased significantly after 15 days after flowering (DAF) until reaching a peak at 30 and 35 DAF, and then gradually dropped down at 45 DAF [21]. The duration of the seed development influences oil content and fatty acid composition as well as genotype and environment. The maximum crude fat (CF) mass fraction was found in *S. tonkinensis* seeds after methyl jasmonate (MJ) application at 70 DAF and the ACC activity displayed a similar trend to that of the CF mass fraction [22]. The high oil content in this study was seed stage 4 WAF, which was consistent with the high expression of the *acc*B gene in mature seeds. The accumulation of oil is higher at maturity stage which was found in many plants.

CONCLUSIONS

The study of the ACCase gene (*acc*A, *acc*B, *acc*C and *acc*D) expression profiles during *C. viscosa* L. seeds developmental stages from 1 to 4 WAF and found that ACCase levels were directly correlated with seed oil accumulation. These findings are an important first stage to understand the processes that lead to production of high-yielding oil in *C. viscosa* L. and have implications for breeding to maximize oil production.

Acknowledgments

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